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Retinoblastoma protein-interacting zinc finger 1 (RIZ1) participates in RANKL-induced osteoclast formation via regulation of NFATc1 expression

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ABSTRACT

The role of retinoblastoma protein-interacting zinc finger 1 (RIZ1) in receptor activator of NF-κB ligand (RANKL)-induced osteoclast formation was examined in mouse RAW 264.7 macrophage-like cells. The expression of RIZ1 was significantly augmented by RANKL-treated cells. Silencing of RIZ1 with the siRNA significantly reduced the appearance of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells as osteoclasts in RANKL-treated cells. The expression of nuclear factor of activated T cell 1 (NFATc1) as the terminal transcription factor of osteoclast formation was prevented by RIZ1 siRNA. It was suggested that that RIZ1 might participate in RANKL-induced osteoclast formation through the regulation of NFATc1 expression.

1. Introduction

Inactivation of retinoblastoma protein-interacting zinc finger 1 (RIZ1) has been considered as one of the most common abnormalities in several cancers including breast cancer, hepatoma, colon cancer, leukemia, melanoma and osteosarcoma. Retinoblastoma protein-interacting zinc finger 1 (RIZ1) is a member of a nuclear histone/protein methyltransferase superfamily [1] and encodes a zinc finger protein that can bind to retinoblastoma protein and estrogen receptors [2,3]. The RIZ1 protein is reported to play a crucial role in pathogenesis of many cancers including osteosarcoma and breast cancer [4,5]. Both osteosarcoma and breast cancer frequently cause bone metastasis [6]. Tumor microenvironment on the bone surface may impair the normal osteoblast–osteoclasts conversion rate and cause severe skeletal complications [6]. Some oncogenic proteins are involved in osteoclast formation whereas the other ones inhibit it [7,8]. The precise role of oncogenic proteins in osteoclast formation is still controversial. There is no report concerning the involvement of a tumor suppressor RIZ1 in osteoclast formation.

Osteoclasts are multinucleated giant cells responsible for bone resorption [9]. Stimulation of receptor activator of NF-κB ligand (RANKL) with macrophage-colony stimulating factor (M-CSF) induces osteoclast formation from monocyte/macrophage precursors [10,11]. The binding of RANKL to RANK as the receptor activates TNF receptor-associated factor 6 (TRAF6), which is important for the differentiation, activation, proliferation and survival of osteoclasts [9,12]. Several transcription factors including c-Fos, microphthalmia transcription factor (Mitf), and PU1 are also involved in RANKL-induced osteoclastogenesis. As the terminal transcription factor of osteoclastogenesis, nuclear factor of activated T cell 1 (NFATc1) leads to the osteoclast differentiation [9,13,14]. In the present study, we studied the role of RIZ1 on RANKL-induced osteoclast formation. Here, we report here that RIZ1 may augment the expression of NFATc1 and lead to RANKL-induced osteoclast differentiation.

2. Materials and methods

2.1. Materials

Mouse recombinant RANKL and M-CSF were purchased from PeproTech Inc., Rocky Hill, NJ, USA. A tartrate-resistant acid phosphatase (TRAP) staining kit was obtained from Wako, Osaka, Japan.

2.2. Cell culture

Mouse macrophage-like cell line RAW 267.4 was obtained from the Riken Cell Bank (Tsukuba, Japan) and maintained in RPMI 1640 medium containing 5% heat-inactivated fetal calf serum (FCS)

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(Gibco-BRL, Gaithersburg, MD, USA) and antibiotics at 37 °C under 5% CO_2.Transfection *of* short interfering RNA (*siRNA*)

RIZ1-specific siGENOME SMART pool and a non-targeting siRNA were obtained from Dharmacon (Chicago, IL, USA). RAW 264.7 cells were seeded at a concentration of 2×10^5 cells/well in a 6-well plate in growth medium containing no antibiotic. The detailed transfection method was described elsewhere [15]. Briefly, cationic lipid complexes were prepared by incubating siRNA duplexes with Lipofectamine RNAiMAX (Invitrogen) without serum and then added to the cells. After 4 h of incubation, the cells were washed and replaced with fresh growth medium without antibiotics for at least 18 h to allow recovery, followed by a second round of siRNA transfection. After 4 h of incubation the cells were washed and resuspended in growth medium. The culture medium was removed 6 h after transfection and the cells were further cultured with 2.5 ml of fresh growth medium for 18 h. The cells were again treated with the siRNA as the second round transfection. The efficiency of RIZ1 silencing was analyzed by immunoblotting after 72 h.

2.4. Immunoblotting

The immunoblotting method was described previously [16]. An equal amount of proteins (20 μg) were analyzed by sodium dodecyl sulfate-polyacryl amide gel electrophoresis under reducing conditions and transferred to polyvinylidene difluoride transfer membranes (*Invitrogen*). Membranes were treated with various antibodies at 4 °C for overnight. Rabbit polyclonal antibodies to RIZ1 was purchased from Abcam (Science Park, Cambridge, UK). Antibodies to p38 and the IκB kinase (IKK)-α were purchased from Cell Signaling Technology (Beverly, MA, USA). Rabbit polyclonal antibodies to TRAF6 and NFATc1 were purchased from Santa-Cruz biotechnology (Santa-Cruz, CA, USA). Those antibodies were used at 1:500–1:2000. The immune complexes were detected with horseradish peroxidase-conjugated protein G (eBioscience, San Diego, CA, USA) at 1:5000 for 1 h and the bands were visualized with a chemiluminescent reagent (Pierce). The chemiluminescence was detected by a light capture system analyzer AE6955 (Atto, Tokyo, Japan).

2.5. Reverse transcription (RT)-polymerase chain reaction (PCR) analysis

RT-PCR was performed as described previously [17]. Semi-quantitative RT-PCR was carried out by using access quick RT-PCR system (Promega, Madison, WI, USA). Primer sequence for RIZ1, forward 5′-TCAGAGGGCACAGGCAAGAC-3′ and reverse 5′-ATGCTTCCAGGTTGTCCCCAGA-3′, GAPDH forward 5′-ATGGGG AAGGTGAAGGTCGGAGTC-3′ and reverse 5′-GCTGATGATCTTGAG GCTGTTGTC-3′ were obtained from Invitrogen (Carlsbad, CA, USA). GAPDH were used as an equal loading control. PCR products were analyzed by electrophoresis on 1.5% agarose gel. The gels were stained with CYBR safe DNA gel stain (Molecular probe, Eugene, OR, USA). The 100 bp DNA was used for the size marker.

2.6. Osteoclast formation and TRAP staining

RAW 264.7 cells with cultured with RANKL (100 ng/ml) and M-CSF (25 ng/ml) for 3 days for osteoclast formation [18]. RAW 264.7 cells were transfected with RIZ1 or control siRNA and then used for the osteoclastogenesis TRAP staining was carried out according to the manufacturer's instruction. Briefly, cells were washed with phosphate-buffered saline and treated with a fixation solution at room temperature for 5 min. The cells were washed with distilled water and treated with TRAP-reagent at 37 °C for 20–60 min. After washing with distilled water, the cells were observed under a microscope. The images were taken with a digital camera attached to the microscope. For the frequency of TRAP-positive cells, more than 150 cells/well were counted microscopically.

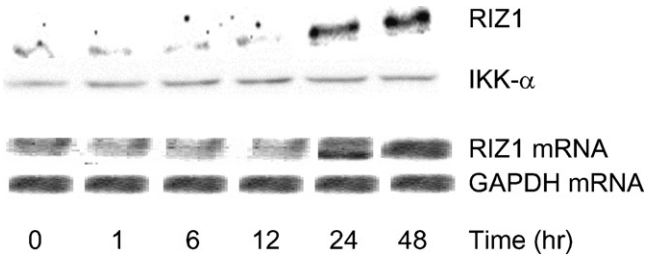

Fig. 1. Expression of RIZ1 in RANKL and M-CSF-stimulated RAW 264.7 cells. Cells were incubated with RANKL (100 ng/ml) in presence of M-CSF (25 ng/ml) for various hours. The expression of RIZ1 protein and mRNA was analyzed by immunoblotting and RT-PCR, respectively. IKK-α and GAPDH was used as loading controls.

2.7. Statistical analysis

Statistical analysis was performed by the Student's *t*-test, with $p < 0.05$ considered significant. All experiments were carried out at least three independent times. Data represent the mean value of triplicates ± standard deviation.

3. Results

3.1. Augmented expression of RIZ1 in RANKL and M-CSF-induced RAW 264.7 cells

RANKL induces the formation of osteoclasts in collaboration with M-CSF [10,11] and positively regulate the osteoclastogenesis through activation of several genes [6,7]. Therefore, the expression of RIZ1 in RANKL and M-CSF-stimulated RAW 264.7 cells was examined (Fig. 1). RAW 264.7 cells were stimulated with RANKL (100 ng/ml) in presence of M-CSF (25 ng/ml) as described previously [18]. The time course of RIZ1 expression was followed and the expression level was determined by immunoblotting. RIZ1 was only slightly expressed in non-treated control cells. Co-stimulation *t* with RANKL and M-CSF significantly augmented the RIZ1 expression at 24 h and the intensity further increased at 48 h. The RIZ1 mRNA expression corresponded to the RIZ1 protein expression. In addition, recombinant M-CSF alone did not augment RIZ1 expression.

3.2. Effect of RIZ1 siRNA on the activation of NFATc1 expression

RANKL activates TRAF6, c-Fos and Ca^{2+} signaling and triggers a sustained NFATc1-dependent transcriptional program during osteoclast differentiation [19]. RANKL-mediated signaling cascades finally induce the expression of NFATc1 as a terminal transcription factor of osteoclastogenesis [19]. Therefore, the effect of RIZ1 siRNA on the regulation of RANKL-induced NFATc1 activation was examined by immunoblotting using anti-NFATc1 antibody. Silencing of RIZ1 with the siRNA remarkably inhibited RANKL-induced NFATc1 activation 3 days after RANKL treatment (Fig. 2), suggesting that RIZ1 might regulate the activation of NFATc1, the master switch for terminal differentiation of osteoclasts. In addition, the RIZ1 silencing resulted in no significant inhibition in the NFATc protein expression 24 h after RANKL treatment.

3.3. No effect of RIZ1 siRNA on the activation of TRAF6 in RANKL and M-CSF-stimulated RAW 264.7 cells

TRAF6 is a crucial docking molecule that mediates the signaling events initiated by RANKL and leads to osteoclast formation